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AD_____

CONTRACT NUMBER DAMD17-97-C-7009

TITLE: Rapid Detection of Arthropod-Borne Pathogens in Mosquitoes

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CONTRACTING ORGANIZATION: Universal Healthwatch, Incorporated
Columbia, Maryland 21045

REPORT DATE: June 1997

TYPE OF REPORT: Final - Phase I

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

9-16-97

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (*Leave Blank*)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1997	Final, Phase1 Nov 11, 1996 - May 10, 1997

4. TITLE AND SUBTITLE
Rapid Detection of Arthropod-borne Pathogens in Mosquitoes

5. FUNDING NUMBERS
DAMD17-97-C-7009

6. AUTHOR(S)
David Bernstein

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Universal HealthWatch Inc.
Columbia, MD 21045

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, MD 21702-5014

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (specific authority). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, MD 21702-5012

12b. DISTRIBUTION CODE
9-16-97

13. ABSTRACT (*Maximum 200 words*)

The increased incidence of malaria and dengue fever around the globe has created a need for more monitoring and surveillance in the field where infected mosquitoes may be encountered.

We proposed to develop and deliver 1,000 one-step "field usable" tests capable of detecting dengue virus, P. vivax and P. falciparum in mosquitoe extracts. We contracted to provide rapid tests utilizing such antibodies and antigens available from the DOD. The success of this program was dependent upon the performance characteristics of the reagents provided.

We screened, selected, and optimized reagents provided by WRAIR in a rapid immunochromatographic test format. In the simple procedure, a test strip is inserted into 0.2 milliliters of malarial mosquito extract in a clear plastic tube. After 5 minutes the strip is inspected for the appearance of a red-purple colored procedural control line. A positive result is indicated by the appearance of a second red-purple line. We prepared several hundred strips employing the selected malarial (P. vivax, P. falciparum) monoclonal antibodies. The sensitivity of these assays is in the range of 12 nanograms per 0.2 ml of circumsporozoite protein. These strips have been placed in labeled foil pouches in 50 test kits along with extraction reagent, controls, and package insert.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Mosquitoes, Arthropod-Borne, Detection, Malaria, Dengue Fever	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

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INTRODUCTION

There is a recognized need for identifying the presence of specific pathogens in the environment. As a result of global warming and globalization of travel and goods, emerging and reemerging infectious diseases are significantly increasing throughout our world. Of particular importance are the spread of arthropod vector borne diseases--particularly those diseases spread by the mosquitoes. The proliferation of mosquitoe-borne diseases such as malaria and dengue fever have been a result of increased resistance to pesticides by mosquitoes, reduced use of pesticides such as DTT, and expansion of mosquito ecological niches due to global warming. At the same time, increased incidence of malarial treatment failures due to resistance to quinine and chloroquinine drugs has created a more dangerous situation.

The U.S. military has had a long history of encountering malaria among its troops. At least one million soldiers suffered from malaria during the Civil War and over 500,000 troops were infected during World War II, primarily in the South Pacific Theatre. By 1963, U.S. soldiers fighting in Viet Nam first encountered chloroquinine resistant malaria

Identification of arthropod-borne pathogens in mosquitoes had previously relied on ELISA methods performed in a well-equipped laboratory among highly skilled personnel. The ELISA requires multiple reagent additions and washings and incubation times of at least four hours. It is unlikely that our military when engaged in the field around the globe would have immediate access to a fully equipped laboratory. This project addresses the need for a rapid (<5 minute turn-around-time), room temperature storage, one step immunochromatographic strip for the detection of arthropod-borne pathogens in mosquitoes which can be used by advancing military teams in the field. Timely identification of the arthropod born disease threat will insure increased compliance with personnel protective recommendations and/or selective vector control efforts.

The present project, to develop rapid, simple tests to detect both malaria and dengue pathogens in mosquitoes can have a major positive impact on control of these diseases in both military and civilian populations. The increased incidence of these diseases around the globe and the need for better monitoring and surveillance has created a need for a simple rapid field test to assess the risk to human health where ever infected mosquitoes may be encountered.

In this Phase I Small Business Research Grant (SBIR), we proposed to develop and deliver 1,000 one-step "field usable" tests capable of detecting dengue virus, P. vivax and P. falciparum from mosquitoes. We contracted to provide such rapid tests utilizing such antibodies and antigens then currently available from the Department of Defense. After the SBIR contract was approved we were informed that these reagents would be provided by Walter Reed Army Medical Center (WRAMC). The success of this program was dependent upon the performance characteristics of the provided antibody reagents and the antigens.

The SBIR contract commenced on November 11, 1996. Principal investigator David Bernstein was informed by DOD that Robert Wirtz, PhD would be the contact person for obtaining antibodies and antigens for the project. Robert Wirtz informed Bernstein that effective December 1, 1996 he will be transferring to Centers for Disease Control in Atlanta, Ga. Dr. Wirtz offered to provide us with reagents before he left and informed us that dengue reagents should be obtained from Robert Putnak, PhD (WRAIR). Dr. Wirtz also informed me that any further requests should be made through Lt Col Ronald Rosenberg (Dept of Entomology, WRAIR). David Bernstein contacted Dr. Putnak and was told that dengue reagents were not available at that time but would be provided by the end of April, 1997.

METHODS

Protein G Sepharose™ Column Purification of moncolonal antibody from Ascites

1. Ascites is centrifuged at 3000 x rpm using bench top centrifuge for 20 min. Supernatant of ascites is filtered through 0.45 μm cellulose acetate filter.

2. A Protein G Sepharose 4 Fast Flow column of 3-5 ml gel [bed volume] is be equilibrated with 10 mM PBS (pH 7.4). The process is continued until the pH of eluted buffer is adjusted to 7.4.

3. A 2-5 ml quantity of the filtered material is applied to the Protein G Sepharose 4 Fast Flow column.

4. The column is washed with 20-30 ml of 10 mM PBS containing 0.02% Na-azide (pH 7.4) to remove unbound contaminants.

5. Elution is done by using 100 mM Citrate buffer [pH 3.0].

6. One ml is collected in each tube containing 100 μl of 1 M Tris-HCl buffer [pH 8.0].

7. Based on OD_{280}, tubes with OD 0.3 and higher are pooled.

8. Dialysis is done against 0.15 M NaCl at 4°C with two changes in 24 hr.

9. Purified monoclonal antibodies used for coating membranes are dialysed against .10 mM PBS pH 7.4 with three changes in 24hr.

10. Monoclonal antibodies used for labeling with colloidal gold are dialyzed against .002M Borax Buffer pH 8.5. with three changes in 24 hours.

11. Dialyzed materials are filtered through 0.2 μm cellulose acetate membrane. Protein content is determined by measuring OD_{280}, calculated protein mg/ml = (OD_{280} X DF) / 1.4 .

Immobilization of capture antibodies on nitrocellulose membrane was done by spotting lines with Camag TLC equipment, followed by desiccation of the nitrocellulose strips.

Optimal pH of colloidal gold sol for labeling with MAb:

A pH-variable adsorption isotherm is performed with the colloidal gold to obtain an optimal pH for labeling with MAb as follows: *Ca.* 1 ml of colloidal gold, at an adjusted pH, e.g., 6.0, 6.5,

7.5, 8.0, 8.5, 9.0, 9.5, and 10.0, is added to test tubes. The pH of the colloidal gold will be adjusted using 0.2 M K_2CO_3, as described above. MAb will then be added to obtain X µg/ml [the amount is determined upon completion of optimization experiment] of colloidal gold. The mixture is allowed to react for 15 min., after which 100 µl of 10% NaCl is added to destabilize the colloid. Thus, the optional pH will be determined which will provide protection against salt destabilization.

Optimal Monoclonal Antibody (MAB) concentration for labeling with colloidal gold:

The optimal MAb concentration for labeling with colloidal gold is obtained by constructing a concentration variable adsorption isotherm, following the procedure described below. The pH of the gold sol is adjusted to optimal pH graph pH variable absorption isotherm with 0.2 M K_2CO_3 using a pH meter. The test involves the following steps: a) addition of 1 ml of unlabeled colloidal gold sol to nine test tubes (1 to 9); b) preparation of a dilution series of MAb by adding the required amount of MAb from a known concentration (100 µg/ml) in test tubes 1 to 9 to obtain final concentrations of 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml of colloidal gold; c) incubation period to react for 15 min; and d) then addition 100 µl of 10% NaCl (w/v) to destabilize the colloidal gold sol and determination of the concentration of MAb that will be sufficient to protect the colloidal gold against salt destabilization. Destabilization should be visualized by color change from light red to blue, which can be quantitated by an increase in absorbance at 580 nm using a spectrophotometer.

Protocol for labeling monoclonal antibodies with colloidal gold particle:

1. Dialysis of purified antibodies is done against 2 mM Borax [pH 9.0] buffer.

2. By measure OD_{280}, protein amount is calculated dividing with factor 1.4 to get mg/ml.

3. Preparation begins with 100 mL of gold-colloidal suspension and pH is adjusted to an optimal with 0.2M K_2CO_3 and OD_{520} will be checked

4. Antibodies are added slowly to gold sol while stirring gently, to a final concentration of X µg/mL [the amount will be determined upon completion of optimization experiment].

5. A 100 µl of labeled colloidal-gold is added with 50 µL of 10% NaCl in a separate tube to see change in color purple to blue. If stable, remains unchanged.

6. The reaction is allowed to equilibrate for 10-15 minutes at RT.

7. BSA [adjusted to pH of 8.5-9.0 using NaOH, filtered through 0.22 µm membrane] is added while stirring to a final concentration of 1%. A 30 minutes time is allowed to equilibrate.

8. The colloidal gold is centrifuged at 9,000 X g for 45 min at 4°C.

9. The soft pellet is resuspended in remaining volume after aspirating off supernatant.

10. The tube is refilled with original volume with 20 mM Tris-buffer .

11. Storage is done overnight at 4°C.

12. Centrifugation is performed under the same conditions and repeated two more times.

13. After final spin, the pellet is resuspended in about 10 mLs of Tris-BSA buffer. The final product is filtered through 0.22 μm.

14. The OD_{520} is checked. If solution has an OD reading below 5.0, it centrifugation is repeated again at 9,000 X rpm for 45 minutes, about 10 mLs of buffer is added and OD at 520nm is checked until in an acceptable range is reached.

15. Labeled antibodies are placed in a light protected area and store at 4°C.

16. A 10 μl amount is spotted per conjugate pad.

17. The conjugate pad is air dried for about 20 minutes and kept in -70°C for 1 hr.

18. Lyophilization is performed on the conjugate pad overnight.

19. Strips are stored in a desiccator in the dark.

Selection of specific antibodies and strip construction:

A testing matrix is utilized to test combinations of specific monoclonal antibodies directly in a chromatographic strip format versus high concentrations of positive and negative controls diluted in an appropriate diluent.

Each polyclonal antibody is purified into an IgG fraction and diluted in Phosphate buffered Saline [PBS] buffer pH 7.4 containing 0.05% Sodium Azide.

Nitrocellulose membranes are spotted with each diluted monoclonal antibody using Camag TLC spotting equipment. Chromatographic strip devices are first constructed by fixing the nitrocellulose to adhesive backed mylar plastic. Absorbent paper is then fixed to the upper portion of the strips and a sampling membrane are fixed to the lower portion of the strip. These backed membranes are then cut into 4 mm wide strips. Each monoclonal antibody is labeled with colloidal gold. Each of the specific mouse monoclonal antibodies is diluted in Borax buffer. Positive control antigens are diluted with extraction reagent in polystyrene plastic tubes. The chromatographic strips are placed in the tube to allow the fluid to flow across the nitrocellulose membrane. After all the fluid in the tube enters the chromatographic strip the colloidal gold diffuses up the strip. All of the chromatographic strips are examined for the best appearing reactions (positive is positive, negatives

are negative).

Once the monoclonal antibodies are evaluated, a larger amount of antibody is purified from the ascites using Protein G Sepharose [Pharmacia] chromatographic column. The purified antibodies are then labeled with colloidal gold particles following appropriate isotherm optimizations. The colloidal gold labeled monoclonal antibodies are diluted in Borax buffer and applied to the lower sample portion of a test strip, frozen and lyophilized. The prepared test strips are then tested for sensitivity and specificity against positive and negative control samples.

The test strips using the best monoclonal antibody combinations are prepared and placed in aluminum foil pouches with appropriate desiccants and sealed.

MALARIA REAGENTS

On November 26, 1996, Dr. Robert Wirtz shipped us the following reagents for our use in the development of rapid malaria tests for the detection of Plasmodium vivax and Plasmodium falciparum circumsporozoites:

MONOCLONAL ANTI MALARIA CIRCUMSPOROZOITE REAGENTS

monoclonal antibody	identification	concentration mg/ml
Pf2A10	474/1D6	2.8
Pf1B2.2	93-3-5	8.0
Pv-210	NSV#3	7.1
Pv-247	1G12.1	7.4

We received approximately 10 mg of each of the above monoclonal antibodies.

Dr. Wirtz at the same time provided the following antigens:

antigen	identification	circumsporozoite protein concentration mg/ml
Pf-R32tet32	104189	0.74
Pv-210	105232	1.0
Pv-247		0.025

Utilizing monoclonal antibodies and antigens described above provided by WRAIR for malaria

(P. falciparum and P. vivax circumsporozoites), we have screened, selected, and optimized these reagents to provide Dipstick strip tests in a rapid immunochromatographic format.

The immunochromatographic strip test that we proposed to develop for detection of malarial or dengue fever antigens was similar in appearance to the rapid immunochromatographic strip test that we had previously developed for Vibrio cholerae infection. These tests are comprised of an extraction step wherein an extraction reagent is added to a sample to dilute and extract target antigens from an organism or virus, and a second step of the test comprised of placing an immunochromatographic strip into the antigen extract solution. The immunochromatographic strip is comprised of a mylar backed nitrocellulose strip in which is immobilized specific capture antibodies to the target analyte. Colloidal gold labeled specific antibody to the target analyte is dried onto a porous cellulose filter paper pad in which its distal portion is in direct contact with the proximal end of the nitrocellulose. The proximal end of an cellulose absorbant pad is in direct contact with the distal end of the nitrocellulose to act as a reservoir for fluid migration. A procedural control line is constructed by immobilizing goat anti-mouse immunoglobulin onto the nitrocellulose distal from the target analyte capture line. The procedure involves placing the constructed immunochromatographic strip with colloidal gold side downward into a tube containing the extracted antigen solution (approximately 200 microliters). The extracted antigen solution migrates up the chromatographic strip and solubilizes the colloidal gold conjugate which then moves with the solvent front through the porous nitrocellulose strip. As immune complexes formed between the antigen and the colloidal gold labeled specific antibody migrates to a line of the specific antibody immobilized on the membrane. The immobilized antibody captures the antigen-gold-labeled antibody complex which forms a distinct pink to red colored line. If antigen is absent or below a detectable thresshold then no color appears in the detection area. As the colloidal gold labeled antibody continues to migrate along the nitrocellulose strip, it becomes captured by the immobilized anti mouse IgG line and appears a distinct pink to red colored line. The reactions are complete after 5 minutes and a test result is determined by visually observing the presence of two colored lines for a positive result or the presence of one colored line for a negative result.

RESULTS

The following summarizes the progress that was made in this project leading up to the delivery of rapid tests for P. falciparum and P. vivax:

PREPARATION OF COLLOIDAL GOLD

Raw colloidal gold reagent (20nm) was prepared by boiling a solution of tetrachloroauric acid with sodium citrate. After boiling for 2-4 hours, the colloidal gold sol was allowed to cool and an optical density at 520nm was determined and then adjusted with water to a final optical density at 520 nm to 1.2 .

COLLOIDAL GOLD LABELING

The final optimal pH conditions for labeling colloidal gold with monoclonal antibodies Pv-247

Pf-2A10 was determined to be between 8.5-9.0. The optimal protein load of both of these monoclonal antibodies was determined to be 20 micrograms of protein for each milliliter of unlabeled colloidal gold. A fifty milliliter pilot batch of each of these monoclonal antibodies was prepared and concentrated to an OD_{520} of 5.20, resulting in a final volume of 4.8-5.0 milliliters of these colloidal gold labeled monoclonal antibodies. A 10 microliter aliquot of each of these two colloidal gold labeled monoclonals was applied to a porous cellulose absorbent paper and then dried in vacuo.

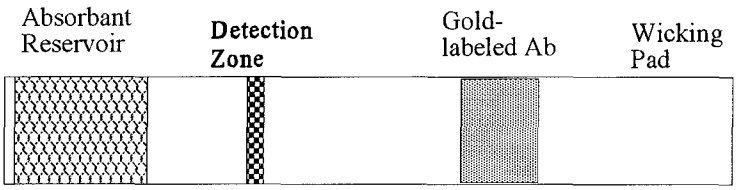
ANTIBODY COATING OF NITROCELLULOSE

Each of the four anti sporozoite monoclonal antibodies [Pf-2A10, Pf-1B2.2, Pv-210, Pv-247] provided to us was diluted into .05M borate buffer pH 8.2. Approximately 1.5 microgram protein (1 mg/ml) was applied as a line to the nitrocellulose. Each of the antibodies were spotted and the nitrocellulose strip was then allowed to dry at room temperature for at least 1 hour. The nitrocellulose strips are kept dessicated until ready for strip assembly.

PREPARATION OF TEST STRIPS

The first strips assembled were made without the procedural control line and

were assembled as follows:



Test strips were assembled as follows: At the middle of an adhesive backed Mylar strip was affixed a smaller antibody treated nitrocellulose strip. In contact with the distal end of nitrocellulose strip on the Mylar strip was affixed an absorbent reservoir pad. In contact with the proximal end of the nitrocellulose strip was affixed the colloidal gold reagent pad and an absorbent wicking pad.

TEST OPTIMIZATION

We screened various combinations of monoclonal gold labeled antibodies and monoclonal capture antibodies for both *P. falciparum* and *P. vivax* sporozoites. The best combination for detection of *P. falciparum* has been with Pf-2A10 monoclonal antibodies for both detection (colloidal gold label) and capture (immobilized on the nitrocellulose membrane). Similarly, the best combination for detection of *P. vivax* has been with Pv-247 monoclonal antibodies for both detection and capture. For testing purposes, we diluted the *P. falciparum* positive control antigen (Pf-R32tet32 104189, 0.74 mg/ml

and capture. For testing purposes, we diluted the *P. falciparum* positive control antigen (Pf-R32tet32 104189, 0.74 mg/ml circumsporozoite protein) and the *P. vivax* positive control antigen (Pv-210 105232, 1.0 mg/ml circumsporozoite protein) in water.

TEST PROCEDURE

The test procedure was as follows:

1. Pipette 200 microliters of antigen dilution into a clean 10 x 75mm plastic test tube.
2. Insert 1 test strip into the test tube and allow the reactants to flow up the length of the test strip.
3. After 5 minutes inspect the test strip for a distinct red band which is indicative of a positive result.

TEST RESULTS

Plasmodium falciparum detection strip could detect as little as 41 ng (per 200 microliters of sample). Some cross reactivity with *Plasmodium vivax* antigen was observed. *Plasmodium vivax* detection strip could detect as little as 50 ng (per 200 microliter sample). A background of colloidal gold reagent could be observed with diluent alone and this made interpretation of endpoints more difficult.

ANTIGEN	CONCENTRATION ng/ml	P. falciparum	P. vivax
Pf-R32tet32	185	3+	N (background)
"	92	2+	N (background)
"	41	1+	N (background)
"	20	N (background)	N (background)
Pv-210	200	1+ (weak)	4+
"	100	N (background)	3+
"	50	N (background)	1+
"	25	N (background)	N (background)
Diluent alone	0	N (background)	N (background)

Experiments were performed to determine the best pair of capture and detection antigens. The best combination for detection of *P. falciparum* has been with Pf-2A10 monoclonal antibodies for

both detection [colloidal gold conjugate] and capture [immobilized on the nitrocellulose membrane]. Similarly the best combination for detection of *P. vivax* has been with Pv-247 monoclonal antibodies for both detection and capture. We prepared new batches of gold conjugate and prepared test strips with the best capture antibodies.

We performed sensitivity studies using the newly made batch of strips. The *P. falciparum* strips were slightly more sensitive than *P. vivax* strips. Slight background was observed with negative controls.

ANTIGEN	CONCENTRATION ng/ml	P. falciparum	P. vivax
Pf-R32tet32	185	4+	N (background)
"	92	1-2+	N (background)
"	41	1+	N (background)
"	20	N (background)	N (background)
Pv-210	200	1+ (weak)	3+
"	100	N (background)	2+
"	50	N (background)	1+
"	25	N (background)	N (background)
Diluent alone	0	N (background)	N (background)

EXTRACTION REAGENT

As a result of background problems with the strips, we began an investigation using various blocking reagents including the following: Tween 20, Triton X-100, NP-40, chondroitin sulphate, sodium heparin, polyanetholsulfonic acid, and bovine serum albumen. The most promising blocking agent appeared to be polyanetholsulfonic acid (PASA) and this reagent was chosen for further testing. We prepared various extraction solutions to resolve the problem of slight background observed with previous preparation of strips. The reagents include different concentration of Borate buffer, buffer with heparin and polyanetholesulfonic acid [PASA]. Satisfactory results was obtained with PASA solution at a concentration of 0.3% [as shown in table below]:

INTENSITY OF BACKGROUND OBSERVED

Concentration PASA (%)	P. falciparum strip	P. vivax strip
0.1%	Strong background	Strong background
0.2%	Slight background	Slight background
0.3%	Clear	Clear
0.4%	Clear	Clear

The new batch of *P. falciparum* strips and *P. vivax* strips did not produce any background reaction when appropriate extraction solution [containing 0.3% PASA] was used.

TEST PROCEDURE & RESULTS

1. Positive control antigens for each strain are diluted in extraction solution containing 0.3% PASA.

2. A 200 μl of the sample is added to a test tube.
3. Test strip is placed into tube containing sample.
4. After 5 minutes the strip is inspected for formation of a purple-red band or a clear strip for negative sample.

A test kit for demonstration purposes containing 20 test strips for malaria (P. vivax, and P. falciparum) was prepared for presentation at a scheduled technical meeting on the 14th March, 1997 at Walter Reed Army Institute of Research (WRAIR) with Lt. Col Ronald Rosenberg and Robert Putnak.

A comparison of reactivity of both falciparum and vivax malarial strips was performed to demonstrate that the nonspecific reaction was removed and the specific reactivity of the antibodies was not significantly diminished.

Sample	P. falciparum strip	P. vivax strip
Extraction reagent (0.3%PASA)	Negative	Negative
P. falciparum 370ng/ml	4+ positive	negative
P. vivax 500ng/ml	Negative	3+ positive

The kit contains:

1. 20 test strip for each [pouched in foil with dessicant]
2. Positive control [approximately 350 ng/ml each] for both *P. falciparum* & *P. vivax* strips
3. Extraction solution

On 3/14/97 David Bernstein and Diane Carter visited Lt Col Rosenberg at building 40, WRAIR and were introduced to Captain Miguel Quintana. Diane performed the malaria test procedures. At the meeting we began to understand the process for collecting mosquito samples in the field. We discussed the methodology used by the entomology group at Walter Reed to collect a mosquito sample and homogenate. The procedure is as follows: Only human eating mosquitoes are of interest, therefore an individual wears short pants. When a mosquito alights on a leg, a mouth suction device (comprised of a mouth piece, rubber tubing, and a glass tube having as screen at one end) is used to collect the mosquito. The mosquito becomes trapped in the glass tube and is then blown out into a round cardboard container with a screen on top. The cardboard container is then placed on ice or in a refrigerator to put down (kill) the captured mosquito. Then surgery can be performed on the mosquito to remove the salivary glands and perform a microscopic observation for the Plasmodium circumsporozoites. The remains of the mosquito containing the gut is placed into an Eppendorf tube (1.5 ml) to which they would add the blocking buffer used in ELISA testing. A cone-shaped plastic pestle is attached to a Dremel drill and the mosquito is homogenized. At a laboratory, each Eppendorf tube is centrifuged to pellet the debris and the supernatant is used in an ELISA. We have had some success in the laboratory in diluting the circumsporozoite in 0.3% polyanetholsulfonic acid, but we do not know if this diluent will successfully release the circumsporozoite in the homogenate. Therefore, I suggested that experiments be performed with various buffer combinations. Lt Col Rosenberg was to provide malaria infected mosquitoes to us for these experiments. We suggested combining P. vivax and P.falciparum into a single test strip but were concerned because of some cross-reactivity with one of the reagents. Captain Quintana said that it would be a rare event for a mosquito to be simultaneously infected with both falciparum and vivax and therefore a combined test would be very useful.

As a result of the meeting we recognized that an important part of the assay for antigen detection in mosquitos will require homogenization into a buffer. Further experimentation will be required to determine if our buffer will adequately release the circumsporozoites. Experimentation would also have to be performed on the homogenization process to determine the appropriate speed (rpm) of the homogenizer, time, and pressure). It is our understanding that centrifugation is required of the homogenate before the ELISA is performed. It is our belief that the test strips could be placed directly in the homogenate without requiring any centrifugation step.

Although we have had some success in the laboratory in diluting the circumsporozoite in 0.3% polyanetholsulfonic acid, but we do not know if this diluent will successfully release the circumsporozoites from a mosquito homogenate. Further experimentation will be required to determine the best buffer combinations for maximum extraction of circumsporozoites for rapid testing.

At our March 14th 1997 meeting with Dr. Ronald Rosenberg at Walter Reed Army Medical Center, we received a better understanding of the current practice for obtaining mosquitoes for testing. We recieved a plastic eppendorf tube and a plastic pestle used to homogenize the mosquito(es) and extract the antigens into a fluid phase. Suggestions were made to construct an

eight well testing apparatus as an array of wells in the shape of the eppendorf tube in which mosquito(es) could be placed and homogenized. We wanted to perform experiments with infected and noninfected mosquito(es) to determine the optimal extraction procedure. We wanted to determine if our exraction reagent could be used to extract the malarial antigens into an eppendorf tube to which a test strip could be added directly and a test result obtained without any further processing and dilution. Unfortunately, we were informed that infected mosquito(es) are not available, but we could obtain uninfected mosquito(es) for testing. We tested the antigens provided diluted in our extraction reagent and waited for receipt of uninfected mosquitoes,

SENSITIVITY TESTING

Malaria P. falciparum and P. vivax test strips were prepared as previously described except that a procedural control line incorporated. Essentially 300ug of goat anti mouse IgG was spotted as a line distal to the specific capture area. After a test is performed, if the procedural control is positive, it indicates that the immobilized antibodies have not deteriorated and the test result is valid. If the procedural control line does not produce a colored line after the test is performed the result would be considered invalid.

Experiments were performed to determine the sensitivity of the (Pv) and (Pf) malaria test strips. **Antigens Pf-R32tet32** (0.74mg/ml) and **Pv-210** (1.0mg/ml) were diluted in extraction reagent containing 0.3% polyanetholsulfonic acid, .05% sodium azide. Antigens were diluted in a range from 1:2000 to 1:64000 and tested.

TEST PROCEDURE

1. Positive control antigens for each strain are diluted in extraction solution containing 0.3% PASA.

2. A 200 μl of the sample is added to a test tube.
3. Test strip is placed into tube containing sample.
4. After 5 minutes the strip is inspected for formation of a red-purple positive control line and either a red-purple line in a positive test or a clear zone in a negative sample.

TEST RESULTS

The results of testing are summarized in the following Table:

SENSITIVITY AND SPECIFICITY OF TEST STRIPS

SAMPLE Dilution	Concentration	Pv antigen vs Pv247 STRIP	Pf antigen vs Pf2A10	Control line
1:2000	500ng/ml	4+	4+	1+
1:4000	250ng/ml	3+	3+	1+
1:8000	125ng/ml	2+	2+	1+
1:16000	62 ng/ml	2+	1+	1+
1:32000	31 ng/ml	negative	negative	1+

4+ = very strong distinct reaction 3+ = strong reaction 2+ = very distinct reaction 1+ distinct reaction negative = no reaction

We prepared several hundred strips employing the selected monoclonal antibodies. The sensitivity of the assay appears to be in the range of 60 ng/ml of circumsporozoite protein. These strips have been foil pouched and labeled and placed in test kits containing 50 tests each. These kits will be delivered with the final report to Fort Detrick.

DENGUE VIRUS REAGENTS

In the previously described March 14, 1997 meeting at Walter Reed Army Institute of Research Dr. Putnak was scheduled to meet with us, but he had evidently forgot about our meeting and he prematurely left the building . Dr. Putnak was supposed to provide reagents and literature for dengue virus detection in mosquitoes at that time.

I communicated to Dr. Bob Putnak in March and was informed that we would receive monoclonals to dengue virus by March 25, 1997 and inactivated dengue virus by the end of April, 1997.

In late April, we received 68 mouse ascites samples from Dr. Robert Putnak. A list of the ascites provided is attached as an appendix. In anticipation of receiving dengue virus antigens, we began purifying the antibody from each ascites sample using protein G sepharose affinity chromatography. Further processing of the purified antibodies will require dengue virus antigens to determine reactivity. We also had requested ELISA information on the performance characteristics of these antibodies, and have not yet received such information. We were expecting delivery of dengue virus antigen from Dr Putnak by the end of April, 1997, but have not received any of these materials. This delay has prevented us from providing dengue virus test strips in a timely fashion.

DISCUSSION

There were several objectives that were proposed in this SBIR Phase I. The first objective was to develop a rapid immunochromatographic strip test format that could detect as few as 100 sporozoites in less than 15 minutes. The antibodies and antigens provided by Dr. Wirtz had previously been used in the development of an ELISA methodology. In the ELISA methodology reported 100 sporozoites could be detected which was equivalent to a protein level of 100pg protein antigen per 200ul. We had received P. falciparum positive control antigen Pf-R32tet32 (0.7mg/ml) and P. vivax positive control antigen Pv-210 (1.0 mg/ml) for P. falciparum. The R32tet32 is a purified P. falciparum circumsporozoite recombinant construct that contains 30 Asn-Ala-Asn-Pro and two Asn-Val-Asp-Pro tetrapeptide repeats fused to 32 amino acids derived from the tet region of the PAS1 plasmid. The P. Vivax 247 variant antigen is a CS positive control peptide comprised of (Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly)$_3$ glutaraldehyde conjugated to boiled casein. We were able to achieve a sensitivity of 40-50ng/ml or 8-10pg protein per 200ul with each of these control antigens. The target sensitivity of the project was to be able to detect 100 sporozoites in a pool of extract from 25 mosquitoes. In the original ELISA tests, mosquitoe extracts are diluted 1:4 in a blocking buffer (PBSpH 7.4, 0.5% NP-40After delivery of the test strips, further testing should be performed with specific concentrations of P. falciparum and P. vivax circumsporozoites to determine exact analytical sensitivity of the test. Initial testing of P. falciparum and P. vivax test strips resulted in some background reactions with the antibody spotted line in addition to some cross reactivity of the P. falciparum test strips with P. vivax positive control antigens. With the addition of polyanetholsulfonic acid blocking reagent the nonspecific reactivity and cross reactivity was abolished. The target goal for the assay was to be able to detect 100 sporozoites in a pool of 25 mosquitoes.

We received approximately 10 mg of each of the monoclonal antibodies used to develop the rapid strip tests. After purification and dialysis approximately 70% of the protein was recovered. Purified monoclonal antibody proteins were used to prepare colloidal gold antibody conjugate and as a capture antibody on the nitrocellulose membranes. It was determined that optimal binding of antibody was at 20ug protein per 1.0 ml of colloidal gold. After colloidal gold was concentated to an optical density of 5.0 at 520nm , 10ul of antibody at 2.0 mg protein/ml were spotted per test. This results in the consumption of approximately 5ug/test. After all the consumption of reagents in optimization, we were left with only enough material to produce 600 tests for P. falciparum and P. vivax.

We did not receive the monoclonal antibodies for dengue virus in a timely fashion and this delayed the progress for a dengue virus test. We did receive 69 ascites samples in April and we proceeded to purify the antibodies from the samples. At the completion of the study, we had not received any dengue virus antigens and have been unable to move forward without this reagent.

We look forward to an evaluation of the test strips being delivered. Stability of these strips are for a minimum of 1 year based on our experience with similar tests we have developed for V. cholerae and E. coli 0157 which all utilized dried immobilized labeled antibodies and capture antibodies stored in a dry foil pouched environment.

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APPENDIX

DENGUE (front to back - left to right)

DESIGNATOR NO.	DATE	REFERENCE NO.
D6-8D2-1	8-15-96	552-27
D3-4B10-3-11	1-16-87	552-31
D2-8B9-9	1-16-81	552-31
D6-1B7-5	9-10-93	778-17
D3-2H2-9-15	9-9-86	552-27
D2-8C2-10	3-21-94	778-26
D2-1F1-3	2-15-90	725-13
D1-3G11-9	11-7-96	552-29
D1-2C6-1	12-8-86	552-29
D6-4F12-11	2-15-84	429-15
D7-5F10-7	10-29-86	552-29
D2-7E11-1	12-4-91	725-42
D8-8E9-1		552-39
D7-3E9-8	3-1-90	725-13
D3-2H2-9-15	12-1-93	778-24
D8-2B12-12		552-39
D6-9E7-5	8-15-86	552-27
D2-5C11-6-19	1-20-87	552-31
D1-3H1-5-10	5-29-87	552-39
D8-3D9-5		552-39
D6-9E1-3	3-5-70	725-13
D3-5A2-7	10-29-86	552-29
D8-2B4-2		552-39
D6-14A4-8	8-19-86	552-27
D6-4E5-4	12-10-91	725-44
D3-1H10-6-4	8-25-87	552-47
D4-3F6-9	2-15-84	429-15
D8-1G6-2	1-9-90	725-11

DENGUE (front to back - left to right)

DESIGNATOR NO.	DATE	REFERENCE NO.
D3-5C9-1	11-30-93	778-24
D1-4E6-4	11-25-86	552-27
D3-4H9-7-5	11-30-93	778-24
D8-2B4-10	5-29-87	552-39
D6-1B7-5	9-10-93	778-17
D2-13E7-9-10	6-20-84	429-26
D6-14A4-4	2-14-84	429-15
D2-13E7-9-10	8-27-86	552-27
D2-11B11-9	6-25-84	429-26
D1-3H5-1-21	1-9-90	725-10
D6-16C7-4	9-26-86	552-27
D2-8C2-10	3-21-84	778-26
D1-4E5-6-5	10-24-87	552-29
D6-8D2-1	3-4-85	429-41
D8-6DZ-7		552-39
D2-13A1-9	1-20-87	552-31
D4-2F3-9-10	9-16-86	552-27
D1-4H10-3		429-40
D6-16C7-4	2-17-89	429-15
D2-113B6-6	1-16-87	552-31
D6-4F12-11	9-8-86	552-27
D3-4G3-2-13	2-2-87	552-31
D6-17H7-2	9-8-86	552-27
D1-4G2-4-15	8-31-87	552-47
D1-2G4-4-7	11-5-86	552-29
D1-4E5-4	8-31-87	552-47
D2-13E7-9-10	6-20-84	429-26
D6-17H7-2	6-25-84	429-26

DENGUE (front to back - left to right)

DESIGNATOR NO.	DATE	REFERENCE NO.
D3-1A2-6	6-27-84	429-26
D6-15H5-8	12-1-93	778-24
D6-9E1-3	3-19-85	429-42
D1-3H1-5-10	5-29-87	552-39
D3-5B2-12	2-2-87	552-31
D3-1H10-6-7	1-9-90	725-10
D1-4H7-9D	1-16-87	552-31
D6-14E9-7	12-8-93	778-24
D7-6B6-10	10-29-86	552-29
D6-9E1-5	8-15-86	552-27
D1-1B10-3	1-16-87	552-31
D4-2F3-10	2-14-84	429-15

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